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High-molecular-weight protein hydrodynamics studied with a long-lifetime metal-ligand complex

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Abstract

[Ru(2,2'-bipyridine)₂(4,4'-dicarboxy-2,2'-bipyridine)]²⁺ (RuBDc) is a very photostable probe that possesses favorable photophysical properties including long lifetime, high quantum yield, large Stokes' shift, and highly polarized emission. In the present study, we demonstrated the usefulness of this probe for monitoring the rotational diffusion of high-molecular-weight (MW) proteins. Using frequency-domain fluorometry with a high-intensity, blue light-emitting diode (LED) as the modulated light source, we compared the intensity and anisotropy decays of RuBDc conjugated to immunoglobulin G (IgG) and immunoglobulin M (IgM), which show a six-fold difference in MW. We obtained slightly longer lifetimes for IgM ($\tau = 428$ ns in buffer) than IgG ($\tau = 422$ ns in buffer) in the absence and presence of glycerol, suggesting somewhat more efficient shielding of RuBDc from water in IgM than in IgG. The anisotropy decay data showed longer rotational correlation times for IgM (1623 and 65.7 ns in buffer) as compared to IgG (264 and 42.5 ns in buffer). Importantly, the ratio of the long rotational correlation times of IgM to IgG in buffer was 6.2, which is very close to that of MW of IgM to IgG (6.0). The shorter correlation times are most likely to be associated with domain motions within the proteins. The anisotropy decays reflect both the molecular size and shape of the immunoglobulins, as well as the viscosity. These results show that RuBDc can have numerous applications in studies of high-MW protein hydrodynamics and in fluorescence polarization immunoassays (FPI) of high-MW analytes.

Keywords

Long-lifetime metal-ligand complex; High-molecular-weight protein hydrodynamics; Light-emitting diode

1. Introduction

Time-resolved fluorescence anisotropy is a powerful technique for investigating macromolecular dynamics [1]. A number of attempts have been made to use fluorescence

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anisotropy decay to study the rotational dynamics of proteins, membrane-bound proteins and other macromolecules. However, the short decay times of about 1 to 10 ns displayed by most fluorophores have been a serious limitation to measure overall rotational correlation times of macromolecules. Information on the rotational motion is usually available over a time scale not exceeding three times the lifetime of the fluorophore, after which there is too little signal for accurate anisotropy measurements. The applications of fluorescence anisotropy decay are now more limited by probe lifetime than by instrumentation.

Long-lifetime metal—ligand complexes (MLCs), which display decay times ranging from 100 ns to more than 10 μ s, have only recently become available [2–4] and show a number of characteristics that make them versatile biophysical probes. Because of the large Stokes' shift, the MLCs do not display significant radiative or nonradiative homo energy transfer [3,4]. Consequently, they do not self-quench even for a protein randomly labeled with several MLCs. In addition, the MLCs display good water solubility and are not prone to self-association [3]. Another favorable property of the MLCs is their high thermal, chemical, and photochemical stability [3,4]. Most MLCs appear to be more stable than fluorescein by a factor of 100 or more [3]. The MLCs display polarized emission, making them useful for microsecond hydrodynamics [3–16]. Finally, if needed, the long lifetimes of the MLCs allow the use of gated detection, which can be employed to suppress interfering autofluorescence from biological samples and can thus provide increased sensitivity [17].

In this laboratory, we introduced the use of long-lifetime MLCs to study microsecond dynamics of proteins [5–11], lipid vesicles [6,12–14], and DNA [15,16] and in clinical applications such as fluorescence polarization immunoassays (FPIs) [8,18–21]. FPI is one of the most versatile and widely used methods for detecting drugs, antigens, and other biological molecules. Because $[\text{Ru}(\text{bpy})_2(\text{dcbpy})](\text{PF}_6)_2$ ($\text{bpy} = 2,2'$ -bipyridine, $\text{dcbpy} = 4,4'$ -dicarboxy-2,2'-bipyridine) (RuBDc) showed the largest fundamental anisotropy among the ruthenium complexes we synthesized [8,10], we believe that this probe is one of the most promising MLCs for studying macromolecular hydrodynamics. RuBDc has been used to monitor the rotational motions of high-molecular-weight (MW) proteins such as concanavalin A [10], human serum albumin [8,10], immunoglobulin (Ig) G [10], and ferritin [10], and in an immunoassay for human serum albumin [8].

To evaluate the usefulness of RuBDc for high-MW protein hydrodynamics, we compared the intensity and anisotropy decays of RuBDc conjugated to IgG (MW= 150,000) and IgM (MW = 900,000), which show a six-fold difference in MW. Measurements were done in the absence and presence of glycerol. For this study, we have used frequency-domain fluorometry with a high-intensity, blue light-emitting diode (LED) as the modulated light source. With this LED, we were able to directly modulate the excitation light up to 100 MHz without the need for an external modulator like a Pockels cell and to obtain very reliable time-resolved intensity and anisotropy decays with simpler and low cost instrumentation.

2. Materials and methods

2.1. Materials

RuBDC was synthesized by the method described previously [10]. IgG and MOPS were purchased from Sigma (St. Louis, MO, USA), and IgM was from Fitzgerald (Concord, MA, USA). Centricon-500 concentrators were supplied by Amicon (Beverly, MA, USA), and Sephadex-G25 M columns were from Pharmacia Biotech (Uppsala, Sweden). Coomassie plus protein assay reagent was provided by Pierce (Rockford, IL, USA), and glycerol was from EM Science (Gibbstown, NJ, USA). All other chemicals were of the reagent grade, and water was deionized with a Milli-Q system.

2.2. Labeling of IgG and IgM with [Ru(bpy)₂(dcbpy)]

IgG was directly dissolved in 0.05 M carbonate buffer (pH 9.2); however, Centricon-500 concentrators were used to concentrate the IgM solution and to change the buffer into 0.05 M carbonate buffer because IgM was supplied as a 1.78 mg/ml solution in 0.05 M Tris, 0.2 M NaCl, pH 8.0. IgG and IgM were labeled with the *N*-hydroxysuccinimide (NHS) ester of RuBDC by adding a 10-fold molar excess of Ru-NHS ester in dimethylformamide to 1 ml of slowly stirred protein solution in 0.05 M carbonate buffer followed by an overnight incubation at 4 °C. The resulting labeled protein was separated from the free dye by passing the solution through a Sephadex G-25 M column, using 10 mM 4-morpholinepropanesulfonic acid (MOPS), 0.85% (w/v) NaCl, pH 7.4. The dye/protein molar ratios of the Ru—Ig conjugates were determined by measuring the absorbance of the Ru—Ig conjugates at 467 nm ($\epsilon_{467\text{ nm}}=12,000\text{ M}^{-1}\text{ cm}^{-1}$) and separately determining the protein concentration with Coomassie plus protein assay reagent. Bovine serum albumin was utilized as a protein standard. The dye/protein molar ratios of Ru—IgG and Ru—IgM conjugates were 3.3 and 27.3, respectively.

2.3. Absorption and steady-state fluorescence measurements

Ultraviolet—visible absorption spectra were measured with a Hewlett-Packard 8453 diode array spectrophotometer. Steady-state intensity measurements were carried out using an SLM Model 8000 spectrofluorometer (Spectronic Instruments, Rochester, NY, USA). The excitation was at 480 nm while emission was observed at 650 nm.

2.4. Frequency-domain intensity and anisotropy decay measurements

Measurements were performed using the instruments described previously [22] and modified with a data acquisition card from ISS (Urbana, IL, USA) [23]. The excitation source was a blue LED LNG992CFBW (Panasonic, Japan) with luminous intensity of 1500 mcd, and an LED driver LDX-3412 (ILX Lightwave, Boscman, MO, USA) provided 30 mA of current at frequencies from 1 to 9.3 MHz. A 450 ± 30 nm interference filter and a 630 nm cut-off filter were used for isolating excitation and emission, respectively. Rhodamine B in water ($\tau = 1.68$ ns) was utilized as a lifetime standard.

The intensity decays were recovered from the frequency-domain data in terms of a multi-exponential model:

$$I(t) = \sum_{i=1}^n \alpha_i \exp(-t/\tau_i)$$

(1)

where the preexponential factors α_i are the amplitudes of each component, $\sum \alpha_i = 1.0$, τ_i are the decay times, and n are the numbers of exponential components. These values were determined by nonlinear least squares analysis as described previously [24,25]. Mean lifetimes were calculated according to equation:

$$\langle \tau \rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} = \sum_i f_i \tau_i$$

(2)

where f_i is the fractional steady-state contribution of each component to the total emission, and $\sum f_i$ is normalized to unity. f_i is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}$$

(3)

The frequency-domain anisotropy decays were also analyzed in terms of the multiexponential model:

$$r(t) = \sum_i r_0 g_i \exp(-t/\theta_i)$$

(4)

where g_i is the amplitude of the anisotropy component with a rotational correlation time θ_i , $\sum g_i = 1.0$, and r_0 is the anisotropy in the absence of rotational diffusion. The total anisotropy (r_0) was a variable or fixed parameter, as indicated in the text. The measurements were performed in air-equilibrated solutions, which caused modest shortening of the lifetimes, or with varying amounts of glycerol. However, the quenching or solvent changes

do not affect interpretation of the anisotropy decays so long as $I(t)$ (Eq. (1)) and $r(t)$ (Eq. (4)) are measured under the same conditions.

3. Results

3.1. Steady-state spectra

Fig. 1 shows the chemical structure of the NHS ester of RuBDc. The absorption and emission spectra of RuBDc conjugated to IgG in buffer are shown in Fig. 2. These spectra are normalized to unity to facilitate comparison. As can be seen in Fig. 2, RuBDc displays both long-wavelength absorption and emission maxima and the large Stokes' shift, with the emission maximum shifting over 180 nm from the absorption maximum. The long-wavelength absorption allowed us to use a simple and inexpensive blue LED as the light source. The long-wavelength emission is valuable because sample autofluorescence is minimized at longer wavelengths. When RuBDc was conjugated to human serum albumin, this probe showed the fundamental anisotropy value (r_0) of 0.23 [8]. This high fundamental anisotropy arises from strong electron-withdrawing property of the dcby ligand, which we interpret as due to directed electron transfer from Ru to dcby. The fundamental anisotropies decreased with less electron-withdrawing ligands, i.e., the monocarboxybiipyridine and the aminophenanthroline ligands [8].

3.2. Time-resolved intensity decays

To determine whether RuBDc can yield useful information about protein dynamics, we performed frequency-domain intensity and anisotropy decay measurements of RuBDc-labeled IgG and IgM in the absence and presence of 30% (v/v) and 60% (v/v) glycerol. The frequency-domain intensity decays of RuBDc-conjugated to IgG and IgM are shown in Fig. 3. One notices that the frequency responses are centered near 0.3–0.6 MHz. The optical output of an LED is easily modulated at kilohertz to low megahertz frequencies. The intensity decays were analyzed in terms of the multi-exponential model according to Eqs. (1)–(3), and the results are summarized in Table 1. In both IgG and IgM, the intensity decays were best fit by a sum of two exponentials. In buffer, the mean intensity decay times for IgG and IgM were 422 and 428 ns, respectively. This result suggests that RuBDc is somewhat more efficiently shielded from water in IgM than in IgG. Similar results were obtained in the presence of glycerol. The mean decay times of IgM in 30% (v/v) ($\langle \tau \rangle = 515$ ns) and 60% (v/v) ($\langle \tau \rangle = 639$ ns) glycerol were slightly longer than those of IgG (509 and 629 ns, respectively, in 30% (v/v) and 60% (v/v) glycerol). As expected, as the concentration of glycerol increases, the mean intensity decay times became longer, probably due to the decrease in the quenching effects of water.

3.3. Time-resolved anisotropy decays

The frequency responses of anisotropy decays of RuBDc conjugated to IgG and IgM are shown in Fig. 4. The best fits of the anisotropy decay data were obtained using the two exponential model, and the results are summarized in Table 2. The long rotational correlation times appear to be consistent with those expected for overall rotational diffusion of Igs, and the short rotational correlation times seem to be due to local motion of RuBDc within Igs. In buffer, the long and short rotational correlation times were 264 and 42.5 ns for

IgG, and 1623 and 66 ns for IgM, respectively. An important point of this result is that the ratio of the long rotational correlation times of IgM to IgG was 6.2, which is very close to that of MW (6.0). This clearly indicates that RuBDc is a good probe for measuring high-MW protein hydrodynamics. The usefulness of RuBDc is further supported by the observation that the long correlation times increased substantially with an increase in glycerol concentration, whereas the short correlation times increased much less with increasing glycerol content. For IgG, the long correlation times increased about 230% and 490% in 30% (v/v) and 60% (v/v) glycerol, respectively. But, only about 120% and 150% increases in short correlation times were observed in 30% (v/v) and 60% (v/v) glycerol, respectively. Similar results were obtained for IgM; however, the difference was smaller than in IgG. The long correlation times showed about 130% and 160% increases in 30% (v/v) and 60% (v/v) glycerol, respectively, and there were about 110% and 130% increases of short correlation times in 30% (v/v) and 60% (v/v) glycerol, respectively. This result is probably due to the fact that the intensity decay time of RuBDc is still short to measure the long rotational correlation times of IgM in glycerol, whereas the correlation times are probably more accurate in the less viscous aqueous solution.

Fig. 4 (top panels) also shows the modulated anisotropy values for RuBDc conjugated to IgG and IgM in the absence and presence of glycerol. In buffer, the modulated anisotropy values for IgM were higher than those for IgG. As expected, we observed higher modulated anisotropy values with an increase in glycerol concentration. Importantly, the modulated anisotropy values for IgM were always higher than those for IgG at the same glycerol concentrations. The analyses in Table 2 were performed with the total anisotropy (r_0) in Eq. (4) as a variable parameter. It is well known that rapid probe motions or lack of adequate time resolution can result in lower apparent r_0 values. We analyzed the anisotropy decay data using a fixed value of $r_0=0.25$, which is known for RuBDc [10]. These analyses resulted in a third short correlation time from 1.4 to 6.5 ns (Table 3). Importantly, the two longer correlation times are comparable for the analyses with r_0 fixed or variable. The correlation times recovered with a fixed value of r_0 are somewhat larger, which is probably due to inclusion of the third correlation time. It is informative to examine the anisotropy decays in the time-domain reconstructed from the frequency-domain data (Fig. 5). The time-domain representation of multiexponential analysis data clearly shows an initial rapid decrease in anisotropy, followed by a much slower loss of the remaining anisotropy at longer times, which we attribute to overall rotational motion of the Igs.

4. Discussion

In the present study, we demonstrated the usefulness of RuBDc, a long-lifetime MLC, for probing high-MW protein hydrodynamics. The most important observation of this report is that the ratio of long rotational correlation times of IgM to IgG approximates that expected from the MW of IgM to IgG. Previously, we were somewhat disappointed with the finding that the rotational motions of the ruthenium MLCs are partially independent of overall rotational diffusion of proteins [10]. Although our data also show considerable local motion of RuBDc (Tables 2 and 3), it is obvious that the slow rotational correlation times reflect the overall rotational diffusion of IgG and IgM.

Rotational motions of biological macromolecules have occasionally been measured using phosphorescence. However, MLCs have several advantages over the phosphorescent probes. In contrast to phosphorescence, the luminescence from MLCs can be measured in the presence of dissolved oxygen. The MLCs are only partially quenched by ambient oxygen, whereas phosphorescence is usually completely quenched. Additionally, there are relatively few phosphorescent probes, but there are numerous MLCs. A number of Os, Re, and Ru complexes have been synthesized and conjugated with macromolecules [3,4]. The use of long-lifetime MLCs as a biophysical probe and in FPI is at present in its infancy, and additional MLCs for microsecond dynamics are yet to be developed.

An important point of the present study is our use of a high-intensity, blue LED as the source of excitation. We were able to obtain approximately 3 to 5 mW of excitation power depending on the LED current. A variety of LEDs including the high intensity UV, blue and green GaN LEDs have been developed as an inexpensive and convenient light sources. LEDs are very inexpensive, about US\$2 to US\$10 a piece, with a few hundreds of dollars for required electronics. The use of LED also allowed us a simpler “turn-key” operation, i.e., less optical adjustments and easier electronic operations. Direct electronic modulation of up to 100 MHz eliminates the expensive and cumbersome electro-optic modulators found in most frequency-domain instruments [26]. Because the frequency responses of overall rotational diffusion of biological macromolecules usually occur at low frequencies due to their big sizes, LED is an ideal light source for measuring microsecond dynamics. We believe that the combination of the use of longlifetime MLCs with LEDs will allow laboratories to perform time-resolved intensity and anisotropy decay measurements with a new generation of simpler and low-cost instruments.

It is of interest to compare the long correlation times recovered from our data with other data available in the literatures. There have been several reports of using anisotropy decays to measure the correlation times of labeled IgG [27,28]. These reports found multiple correlation times consistent with both domain motions and overall IgG rotation. The longer correlation times were near 100 ns. These values are shorter than our mean values in buffer near 270 ns (Tables 2 and 3). This discrepancy can be explained by the shorter lifetimes of the probes used in [27,28], which were near 24 ns, as compared to the lifetime of our MLC probe near 420 ns. Dielectric relaxation measurements also revealed a range of IgG motions with a long correlation time near 200 ns [29]. We also compare our results with those expected from small angle or synchrotron X-ray scattering [30–35]. The scattering data provide measurement of the radius of gyration (R_G) of the proteins. This value is related to the molecular radius (r) of the protein by $r = R_G/\sqrt{0.6}$ [36]. We used these data to calculate the volumes of IgG and IgM using various assumptions (Table 4). The volume of the anhydrous protein can be calculated from its MW and partial specific volume (v) using $V = (MW \times v)/N$ where N is the Avogadro's constant. The volumes can also be calculated using the value of R_G . Finally, we calculated the maximum volume using the volume of a sphere that would enclose the IgG or IgM molecule. Using these volumes, we calculated the expected correlation times (Table 5). These results show that the long correlation times recovered from our data are larger than those from the literature, but smaller than those calculated from the R_G . Our measured correlation times are much smaller than calculated for

a sphere enclosing the proteins, which is the approximate value expected if the proteins were rigid in solution. This comparison suggests that the anisotropy decays measured with our MLC probe reflect both overall hydrodynamic rotation with a contribution of the domain motion, which decreases the long correlation times to values lower than those expected for a rigid sphere.

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Abbreviations:

bpy	2,2'-bipyridine
dcbpy	4,4'-dicarboxy-2,2'-bipyridine
FPI	fluorescence polarization immunoassay
Ig	immunoglobulin
LED	light-emitting diode
MLC	metal–ligand complex
MOPS	4-morpholinepropanesulfonic acid
MW	molecular weight
NHS	<i>N</i> -hydroxysuccinimide
RuBDc	[Ru(bpy) ₂ (dcbpy)](PF ₆) ₂

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Fig. 1.
Chemical structure of NHS ester of [Ru(bpy)₂(dcbpy)]²⁺.

Fig. 2.

Absorption and fluorescence spectra of [Ru(bpy)₂(dcbpy)]²⁺ conjugated to IgG in 10 mM MOPS, 0.85% (w/v) NaCl (pH 7.4) at room temperature. The spectra for [Ru(bpy)₂(dcbpy)]²⁺ conjugated to IgM are identical.

Fig. 3. Frequency-domain intensity decays of $[\text{Ru}(\text{bpy})_2(\text{dcbpy})]^{2+}$ conjugated to IgG (left) and IgM (right). The measurements were done with a modulated blue LED.

Fig. 4. Frequency-domain anisotropy decays of $[\text{Ru}(\text{bpy})_2(\text{dcbpy})]^{2+}$ conjugated to IgG (left) and IgM (right). The excitation was from a blue LED.

Fig. 5. Time-domain representation of anisotropy decays of $[\text{Ru}(\text{bpy})_2(\text{dcbpy})]^{2+}$ conjugated to IgG and IgM.

Table 1Multiexponential intensity decay analyses of [Ru(bpy)₂(dcbpy)]²⁺ conjugated to IgG and IgM

Ig	Glycerol (% (v/v))	τ_1 (ns)	τ_2 (ns)	a_1	a_2	f_1	f_2	$\langle \tau \rangle^a$	$\chi_R^2{}^b$
IgG	0	249	457	0.265	0.735	0.165	0.835	422	3.6
	30	130	517	0.079	0.921	0.021	0.979	509	1.5
	60	120	636	0.065	0.935	0.013	0.987	629	1.2
IgM	0	180	448	0.167	0.833	0.075	0.925	428	2.1
	30	92	522	0.077	0.923	0.014	0.986	515	2.8
	60	255	662	0.134	0.866	0.056	0.944	639	2.4

^aMean lifetimes were calculated using $\langle \tau \rangle = \sum f_i \tau_i$, where f_i is the fractional steady-state contribution of each component to the total emission.

^bThe standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively.

Table 2Multiexponential anisotropy decay analyses of $[\text{Ru}(\text{bpy})_2(\text{dcbpy})]^{2+}$ conjugated to IgG and IgM

Ig	Glycerol (% (v/v))	θ_1 (ns)	$r_0 \times g_1$	θ_2 (ns)	$r_0 \times g_1$	$(r_0 \times g_i)$	χ_R^2 ^a
IgG	0	128	0.110	–	–	–	10.5
		264	0.059	42.5	0.076	0.135	2.9
	30	256	0.122	–	–	–	35.1
		614	0.076	52.0	0.079	0.155	3.1
	60	569	0.148	–	–	–	70.6
		1299	0.110	64.0	0.076	0.186	4.1
IgM	0	444	0.110	–	–	–	37.1
		1623	0.070	65.7	0.067	0.137	2.9
	30	607	0.124	–	–	–	53.9
		2191	0.085	74.0	0.069	0.153	3.6
	60	875	0.153	–	–	–	69.4
		2603	0.113	88.0	0.071	0.182	2.8

^aThe standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively.

Table 3Three exponential anisotropy decay analyses of $[\text{Ru}(\text{bpy})_2(\text{dcbpy})]^{2+}$ conjugated to IgG and IgM^a

Ig	Glycerol (% (v/v))	θ_1 (ns)	$r_0 \times g_1$	θ_2 (ns)	$r_0 \times g_2$	θ_3 (ns)	$r_0 \times g_3$	$(r_0 \times g_i)$	χ_R^2 ^b
IgG	0	284	0.053	54	0.069	1.6	0.128	0.250	2.9
	30	676	0.071	71	0.066	3.6	0.113	0.250	3.1
	60	1350	0.108	74	0.069	3.2	0.073	0.250	4.1
IgM	0	2352	0.063	106	0.056	3.8	0.131	0.250	2.6
	30	5324	0.069	158	0.060	6.5	0.121	0.250	2.3
	60	2691	0.112	94	0.067	1.4	0.071	0.250	2.8

^aThe total anisotropy was a fixed parameter ($r_0 = 0.250$), and the g_i was a variable parameter.^bThe standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively.

Table 4

Molecular volumes (V) of IgG and IgM in buffer^a

Ig	Anhydrous protein		Hydrous protein		Sphere corresponding to R_G ^b		Enclosed sphere ^c	
	MW $\times 10^{-3}$	$\hat{v}$	V^d (nm ³)	V^e (nm ³)	R_G (nm)	r (nm)	V (nm ³)	d_{max} f_{om} V (nm ³)
IgG	150	0.762 [30]	190	328 [32]–399 [31]	6.08 [31]–6.68 [32]	7.85–8.62	2026–2683	21 [33] 4849
IgM	900	0.724 [34]	1082	1609 [34]–1850 [35]	12.10 [35]–12.17 [34]	15.62–15.71	15,964–16,241	36 [34] 24,429

^aMW=molecular weight; $\hat{v}$ = partial specific volume; V = molecular volume; R_G = radius of gyration; r = radius of the sphere corresponding to R_G ; and d_{max} = maximum dimension. The numbers in brackets refer those of references.

^bWe assumed a sphere that corresponds to R_G . The volume of the sphere corresponding to R_G was calculated from r , and the r values were calculated by using $r = R_G/\sqrt{0.6}$ [36]. The R_G values were measured by either small angle [31,32,35] or synchrotron [34] X-ray scattering method.

^cWe assumed a sphere that encloses IgG or IgM molecule. The volume of the enclosed sphere was calculated from d_{max} .

^dCalculated from MW and $\hat{v}$ using $V = (MW \times \hat{v})/N$ where N is the Avogadro's constant.

^eFrom the literatures. The V values were measured by either small angle X-ray scattering method [31,32,35] or hydrodynamic modelling [34].

^fThe value for IgG represents the end-to-end distance of the two Fab arms.

Table 5

Comparison of calculated values for overall rotational correlation times (θ_1) of IgG and IgM in buffer^a

Ig	θ_1 (ns)				
	Anhydrous protein	Hydrous protein	Sphere corresponding to R_G	Enclosed sphere	Observed
IgG	41	71–86	438–580	1049	270
IgM	234	348–400	3453–3513	5284	2000

^aThe rotational correlation times (θ) were calculated according to the Perrin equation $\theta = \eta V/RT$, where η is the viscosity, V is the molecular volume, R is the gas constant, and T is the absolute temperature. The V values were from Table 4.